Limulus kexin: a new type of Kex2-like endoprotease specifically expressed in hemocytes of the horseshoe crab

Shun-ichiro Kawabata*, Kazuko Saeki, Sadaaki Iwanaga

Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812-81, Japan

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Abstract A Kex2-like protease was identified in hemocytes of the horseshoe crab (*Tachypleus tridentatus*), named limulus kexin, and a full-length cDNA was obtained from a hemocyte cDNA library. The deduced amino acid sequence contains 752 residues, composed of five domains with a signal sequence, a propeptide, a catalytic domain, a Ser/Thr-rich domain, and a transmembrane domain. The domain organization is very similar to that of the yeast Kex2 except that limulus kexin does not have a cytoplasmic tail. The catalytic domain exhibits striking sequence identities with those of furins, especially *Drosophila* furin1 (79%). Northern blotting showed specific expression of limulus kexin in hemocytes, suggesting the involvement in proteolytic processing of the granule components of hemocytes.

Key words: Kex2-like protease; Subtilisin-like proprotein convertase; Kexin; Furin

1. Introduction

A number of Kex2-like proteases (kexin family) with different substrate specificities are present beyond species in eukaryotes such as mammals [1-3], insects [4-6], mollusks [7], cnidarians [8], and protochordates [9]. They are Ca^{2+}-dependent serine proteases with a bacterial subtilisin-like catalytic domain and responsible for proteolytic processing of proproteins at dibasic sites. The hemolymph circulating in chelicerata horseshoe crab contains granular hemocytes comprising 99% of the total hemocytes, highly sensitive to bacterial endotoxins, lipopolysaccharides [10]. The granular hemocytes store granular specific proteins into two types of granules, large and small granules, which are released in response to external stimuli such as lipopolysaccharides and Ca^{2+}-ionophore [11-13]. The granular components, which include serine protease zymogens and protease inhibitors participating in hemolymph coagulation, lectin-like proteins, and antimicrobial substances [14-18], have been characterized and cloned. The cDNA sequence analyses indicate that some of them are synthesized as preproproteins with an NH_2-terminal propeptide, in addition to a signal sequence, which linked to their mature proteins through an -Arg-X-Arg/Lys-Arg- motif, suggesting the presence of Kex2-like proteases. We report here the identification of a new kexin-like protease, named limulus kexin, that is expressed specifically in horseshoe crab hemocytes.

*Corresponding author. Fax: (81) (92) 642-2634.
E-mail:skawascb@mbox.nc.kyushu-u.ac.jp

Abbreviations: PCR, polymerase chain reaction

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number D83994.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) and cDNA cloning of limulus kexin

To amplify first-strand cDNA prepared from the poly(A)$^+$ RNA of horseshoe crab hemocytes by PCR, two degenerate oligonucleotide primers corresponding to amino acid sequences (-HGTRCA- for the sense primer and -TWRDMQ- for the antisense primer) of the catalytic domain conserved among the members of the kexin family were designed. PCR was performed in a Perkin Elmer Cetus thermal cycler for 60 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and extension (65°C, 1.5 min). The resulting PCR product of 0.6 kb was subcloned into pBluescript II SK$^+$ (Stratagene) and used as a probe for screening. Plaque hybridization experiments and nucleotide sequence analysis were performed as previously described [11]. One positive clone with a 2.6-kb insert was subcloned into the *Eco*RI site of pBluescript II SK$^+$ and the nucleotide sequence was determined on both strands.

2.2. Northern blotting

Total RNAs were prepared from the various tissues by the acid guanidinium thiocyanate-phenol-chloroform method [19] and the poly(A)$^+$ RNAs were purified using Oligotex-dT30<Super> (Takara Shuzo Co., Kyoto). Northern blot analysis was performed using a digoxigenin-containing cRNA probe prepared from the horseshoe crab cDNA subcloned in pBluescript II SK$^+$ using T7 RNA polymerase and digoxigenin-11-UTP, as described previously [11].

3. Results

3.1. cDNA cloning and sequence analysis

The PCR using the degenerate oligonucleotide primers based on the conserved amino acid sequences in the catalytic domain of the kexin family yielded a band of about 600 bp, which is consistent with the distance between the sequences, and the product was subcloned for further analysis. Sequence analysis revealed that the DNA with 603 bp (designated Pcat) has the significant sequence similarity with the corresponding catalytic domain of Kex2-like proteases. To isolate the full-length clone, a hemocyte cDNA library was screened, using Pcat as a probe. Eleven positive plaques were obtained by screening 5×10^5 phages and the cDNA with the longest insert was sequenced. It contained 2558 bp starting with the ATG codon at nucleotide position 95 and had an open reading frame of 2256 bp (Fig. 1). The open reading frame encoded 752 amino acid residues of a preproprotein containing a subtilisin-like catalytic domain from Asn^{126} to Ala^{413} homologous to the kexin family, indicating that the cDNA codes for a Kex2-like protease of horseshoe crab (designated limulus kexin). A putative signal sequence of 27 residues with a typical hydrophobic core [20] and cleavage at Arg^{112}-Asp^{113} with an -Arg-X-Lys-Arg-motif could yield a mature protein of 640 residues with a calculated M_r=71 040. There are 10 *N*-linked sugar potential sites in the proprotein. Judging from the sequence similarity with subtilisin BPN′ [21], functionally im-

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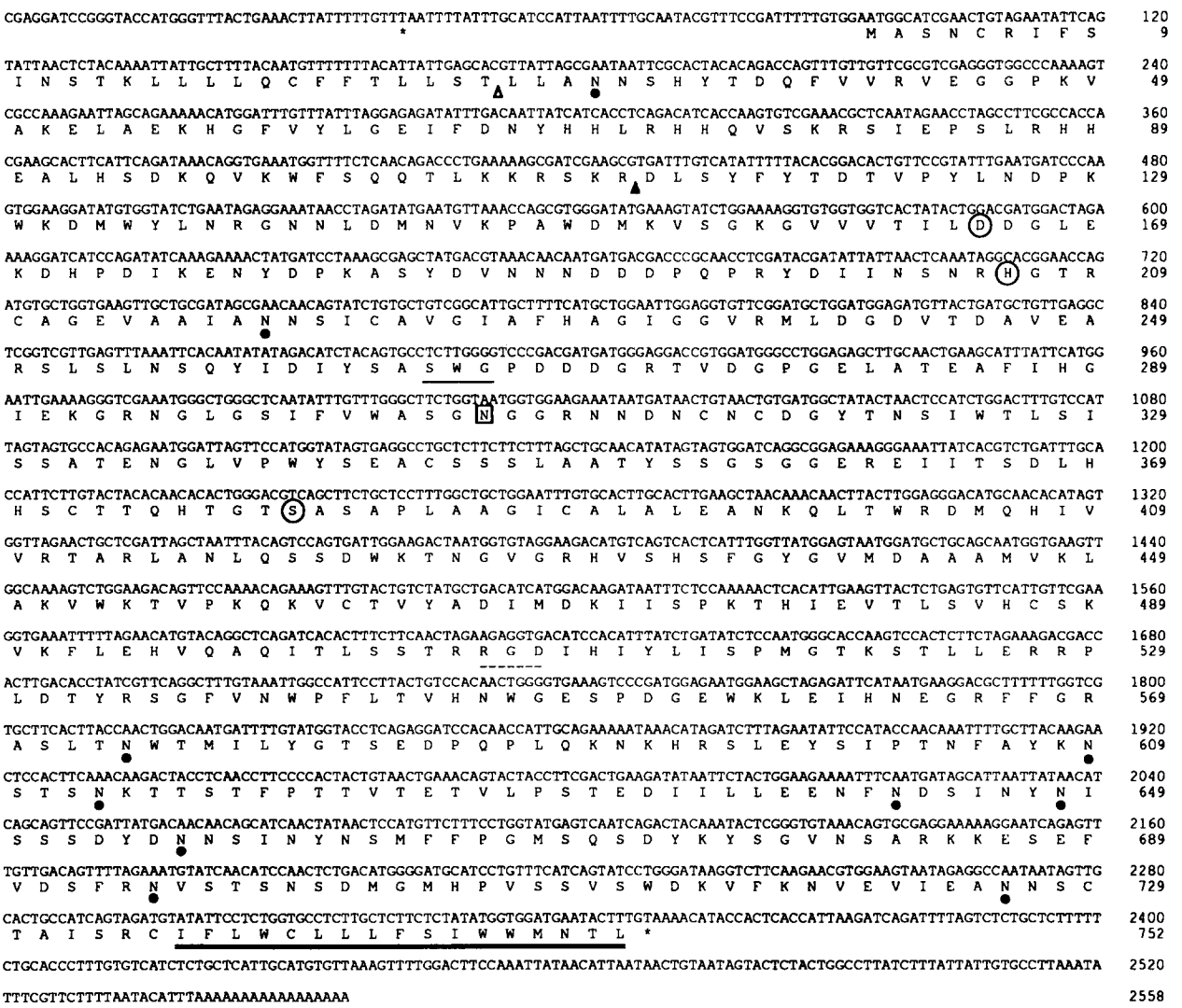

Fig. 1. Nucleotide and deduced amino acid sequences of limulus kexin. Putative cleavage sites for the signal peptide and propeptide are indicated by open and closed triangles, respectively. Potential *N*-glycosylation sites are indicated by closed circles. The active sites Asp, His, and Ser residues are circled, and the Asn residue involved in the oxyanion hole is boxed. The putative transmembrane segment (a double line), the Ser-Trp-Gly sequence (a solid line), and the Arg-Gly-Asp sequence (a dotted line) are also indicated.

portant amino acid residues such as Asp^{165}, His^{206} and Ser^{380} for the catalytic triad, and Asn^{307} participated in the formation of the oxyanion hole, and -Ser^{265}-Trp^{266}-Gly^{267}- for a substrate binding site were well conserved. Another notable structural feature, a potential integrin-binding sequence of -Arg^{507}-Gly^{508}-Asp^{509}- with unknown function in the kexin family, was also conserved at the corresponding positions. Furthermore, there was a domain (Ser^{610} to Ser^{712}) highly occupied by Ser and Thr residues, accounting for 31% in this region. The hydropathy profile indicated that a hydrophobic region is located at the COOH-terminal part from Ile^{736} to Leu^{752}, in addition to the NH_2-terminal signal sequence, suggesting the presence of a transmembrane domain.

3.2. Tissue specific expression of limulus kexin

To determine the size of the mRNA and to investigate tissue specific expression of limulus kexin, Northern blot analysis was performed using poly(A)$^+$ RNAs prepared from hemocytes, heart, hepatopancreas, stomach, intestine, and skeletal muscle (Fig. 2). The expression of limulus kexin was only detected in hemocytes with three different sizes of transcripts, 3.4 kb, 6.4 kb and 7.7 kb, but any sizes of the transcripts were not detectable in other tissues.

4. Discussion

We have found the presence of a Kex2-like protease in horseshoe crab hemocytes and determined the cDNA sequence. The deduced amino acid sequence clearly shows the protein to be a member of the kexin family. Limulus kexin is composed of at least five domains with a signal sequence, a propeptide, a subtilisin-like catalytic domain, a Ser/Thr-rich domain, and a hydrophobic transmembrane domain, as schematically shown in Fig. 3. The domain organization is very similar to that of the yeast Kex2 protease. The catalytic domain of limulus kexin from Asn^{126} to Ala^{413} exhibits striking sequence identities with those of furins, in which *Drosophila* furin1 has the highest homology (79% identity). Limulus kex-

in, however, has lower sequence homology to those of PC2 and PC1/3, suggesting that it is more structurally related to the furin subfamily than PCs. A Ser/Thr-rich domain, which does not exist in other members of the kexin family except for the yeast Kex2 protease, is noticed. In furins and PACE4, this domain is replaced by a cysteine-rich domain [1-3]. Recently, a Ser/Thr-rich segment composed of about 20 amino acid residues was found in *Aplysia* PC1A [7] and *Aplysia* furin2 [22]. The Ser/Thr-rich domain of Kex2 is thought to have *O*-linked oligosaccharide chains [23]. Furthermore, 50% of *N*-linked sugar potential sites are also localized in the domain of limulus kexin, suggesting that this domain is highly glycosylated. Limulus kexin has a hydrophobic segment at the COOH-terminal portion and therefore it may bind to a membrane. It is noteworthy that limulus kexin does not have a cytoplasmic tail unlike the yeast Kex2 protease and furins. Since the cytoplasmic tail of mammalian furins is known to play an important role in their localization at the *trans*-Golgi [24,25], the subcellular localization of limulus kexin may differ from those of furins.

The expression of mRNA of limulus kexin was detected only in hemocytes (Fig. 2). PC1/3, PC2, and PC4 are also expressed in limited tissues and are responsible for proteolytic processing of proproteins stored in granules through a regulated secretory pathway [1-3]. On the other hand, furins are expressed in almost all tissues and such broad tissue expression indicates that furins participate in processing constitutively secreted proteins [1-3]. However, Nagle et al. have suggested that *Aplysia* furin2, a furin-like convertase, is present in secretory granules to process egg-laying hormone-related precursors [22]. Therefore, the limited expression of limulus kexin supports the notion that this protease is involved in the processing of the granular components of the hemocytes through the regulated secretory pathway.

The molecular basis for differences among the three transcripts with the sizes of 7.7 kb, 6.4 kb, and 3.4 kb is currently unknown. They may be generated by alternative RNA splicing of the same primary transcript or they may be products from distinct genes encoding isoforms. The PCR product (Pcat) obtained by amplifying the catalytic region coded for a slightly different sequence from the cloned enzyme (95% amino acid sequence identity, data not shown), indicating the existence of at least one more Kex2-like protease in the hemocytes. The yeast has only one proprotein convertase, Kex2, whereas cnidarian *Hydra*, the most simple organism possessing specialized tissues, has two isoforms of PC1/3-like protease [8], and in mollusk *Aplysia*, five members of the kexin family including PC2- and furin-like enzymes have also been identified [7,22]. Thus, further studies will be required to elucidate a functional role of limulus kexins in horseshoe crab.

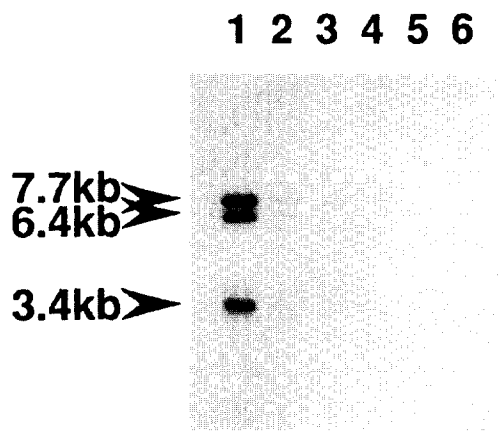

Fig. 2. Northern blot analysis of limulus kexin. Poly(A)$^+$ RNAs (1 μg) prepared from various tissues of horseshoe crab were analyzed using the cRNA probe. Lane 1, hemocytes; lane 2, heart; lane 3, hepatopancreas; lane 4, stomach; lane 5, intestine; lane 6, skeletal muscle.

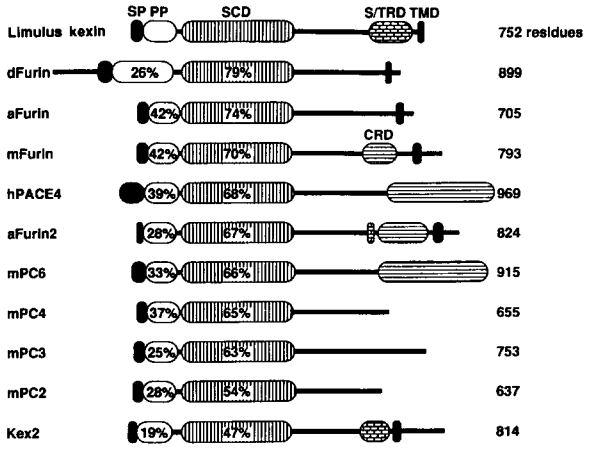

Fig. 3. Domain organization of members of the kexin family. The percentages indicate the sequence identities between the catalytic domains of each protease and horseshoe cab kexin. SP, signal peptide; PP, propeptide; SCD, subtilisin-like catalytic domain; S/TRD, Ser/Thr-rich domain; TMD, transmembrane domain; CRD; Cys-rich domain. dFurin, *Drosophila* furin1 [4]; aFurin, *Aplysia* furin [7]; mFurin, mouse furin [26]; hPACE4, human PACE4 [27]; aFurin2, *Aplysia* furin2 [23]; mPC6, mouse PC6 [28]; mPC4, mouse PC4 [29]; mPC3, mouse PC3 [30]; mPC2, mouse PC2 [31]; Kex2, yeast Kex2 [32].

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